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Structure and Tissue-Specific Expression of the Human Metallothionein I_B Gene

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The human metallothionein (MT) I_B gene (hMT-I_B) is located in a region of human DNA containing at least four tandemly arranged MT genes. As deduced from its sequence, hMT-I_B is likely to encode a functional protein. However, the predicted amino acid sequence differed from the hMT-I amino acid sequence in four positions. Most remarkable was the presence of an additional cysteine. Like other MT genes, hMT-I_B has at least two copies of the metal-responsive element upstream from the transcription initiation site. These elements probably are responsible for the metal responsiveness of the hMT-I_B promoter, leading to inducible expression of fused heterologous genes. Unlike the hMT-II_A and hMT-I_A genes described previously, which are expressed in many different cell types, a high level of expression of the endogenous hMT-I_B gene could be detected only in human hepatoma and renal carcinoma cell lines. Therefore, this is the first MT gene described which exhibits tissue specificity of expression. This specificity is controlled by a *cis*-acting mechanism involving methylation, since incubation of nonexpressing cells with an inhibitor of DNA methylation led to activation of the hMT-I_B gene. In support of this notion, we found that the 5′ flanking region of the hMT-I_B gene was highly methylated in HeLa cells, a nonexpressing cell type, but it was not methylated in a hepatoma (expressing) cell line.

Metallothioneins (MTs) are ubiquituous low-molecular-weight proteins with a high cysteine content and a selective capacity to bind heavy metal ions (15). They can be classified into two major groups, MT-I and MT-II, according to their electrophoretic behavior. The MT-I subgroup can be further resolved into several isoforms, known as MT-I_A, MT-I_B, etc. Although the exact function of MTs is not clear, it is thought that they play a central role in the regulation of trace metal metabolism and in the storage of these ions in the liver. They also may have a protective role against free hydroxyl radicals (39). MT synthesis is induced by heavy metal ions and glucocorticoid hormones at the transcriptional level (18, 20), and by a variety of stressful stimuli, such as the injection of bacterial lipopolysaccharide or UV irradiation. The lipopolysaccharide and UV response suggests that MTs may have a role in the acute-phase response (17), a pleiotropic response to tissue injury and inflammation (25). The role of MTs in the acute-phase response is supported by the fact that MT gene expression is elevated by interferon (8, 9) and interleukin I (21). Interleukin I is known to increase the expression of a number of liver-specific genes during the acute-phase response (25, 31, 35).

The multiplicity of MT forms is confirmed by the fact that in vertebrates they are encoded by a multigene family (12, 22, 34). The human MT (hMT) family consists of at least 12 genes, of which the functional members are located on chromosome 16 (19). There are at least seven functional genes (22, 32; unpublished data) and two mutant MT sequences clustered at 16q22 (27). MT sequences located on other chromosomes are processed pseudogenes (22; unpublished data). The expression and regulation of two of the functional members, hMT-I_A and hMT-II_A, have been studied in detail (20, 32). They exhibit different induction phenotypes mainly due to substantial differences in their respective promoter elements effecting differential transcription control. Despite these differences, expression of both genes has been detected in all cell types tested. In light of the multiplicity of human MT genes, we reasoned that certain functional members may have evolved tissue-specific expression patterns to accommodate a wider-spectrum of control of trace metal metabolism necessary in different cell types. One gene, hMT-I_B, exhibited a differential expression phenotype and was therefore characterized in detail. We found that this gene encodes an apoprotein of the MT-I type, but with an additional cysteine residue at a unique location. The hMT-I_B gene has a functional promoter containing structural features necessary for heavy metal induction. The endogenous gene is expressed in high levels in human hepatoma and renal carcinoma cell lines, while it is practically silent in HeLa cells, B-cell lines, and primary cultures of human fibroblasts. This tissue specificity of expression seems to be determined by a *cis*-acting mechanism involving gene-specific methylation. This is the first example of an MT gene whose expression is limited to selected cell types.

MATERIALS AND METHODS

Sequence analysis. The sequence of the region containing the hMT-I_B gene was determined by the dideoxynucleotide sequencing method (33). Restriction digest fragments were subcloned into M13 DNA (mp8 and mp9) (13). The sequencing strategy is depicted in Fig. 1. The sequence was determined for both strands to eliminate ambiguities.

Construction of recombinant plasmids. To construct the hMTK-I_B gene (an MT-Tk fusion), an 875-base-pair (bp) *Hin*dIII-*Bam*HI fragment containing the 5′ flanking sequence of hMT-I_B was subcloned into pUC8 (40) cut with the appropriate restriction enzymes. The resulting plasmid, pUCI_B5′, was linearized at its unique *Bam*HI site, dephosphorylated, and used as a vector for cloning a 3.6-

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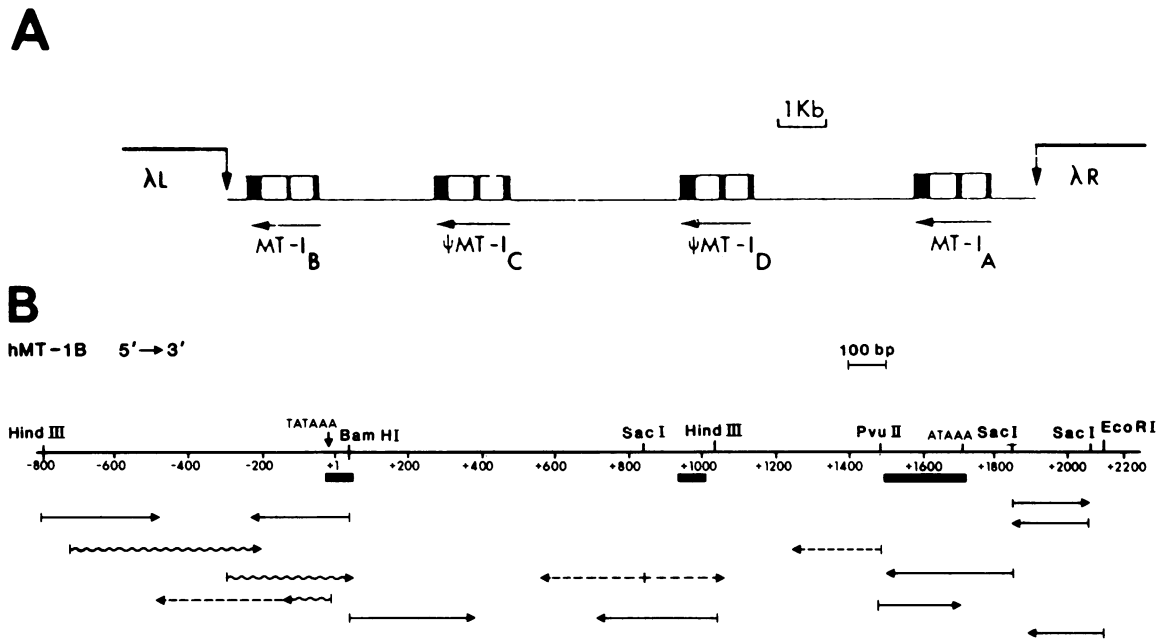


FIG. 1. Structure of the hMT-I_B gene. (A) Physical map of λ hMT-35, which contains four MT-I genes (32), showing the relative position of the hMT-I_B gene within the cluster. The exons are denoted by thick lines. (B) Restriction map of hMT-I_B. The arrows indicate the strategy followed to sequence this gene. Straight arrows are sequences originating at the indicated restriction sites, broken arrows are sequences of clones generated by Bal31 deletions, and broken arrows are sequences of clones generated by random cloning of sonicated DNA.

kilobase *Bgl*II-*Bam*HI fragment containing the coding region and 3′ untranslated region of the herpes simplex virus thymidine kinase (TK) gene (29). The correct orientation was determined by restriction mapping (Fig. 2A). For the chloramphenicol acetyltransferase (CAT) constructions, a *Bgl*II-*Bam*HI fragment containing the CAT structural sequences (11) was cloned into pUC13 and was used as a vector to insert the different hMT promoter/regulatory sequences (see Fig. 6). pUCAT2 is a subclone of the simian virus 40 promoter and the CAT gene from pA10CAT (26) into pUC8.

Cell culture. All but two cell lines used were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Hyclone), penicillin, and streptomycin. The exceptions were the primary human foreskin fibroblasts (a gift from J. Landolph), which were grown in a mixture of Ham F12 medium and Dulbecco modified Eagle medium (1:1) supplemented with 15% fetal bovine serum and the above mentioned antibiotics, and the B-cell lines, which were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Transfections, selection, and CAT assays. phMTK-I_B was transfected into tk^- *Rat*2 cells (1 μg of DNA per plate) by the calcium phosphate precipitate technique (41). tk^+ colonies were selected and maintained in Dulbecco modified Eagle medium plus 10% fetal bovine serum supplemented with 10^{-4} M hypoxanthine, 10^{-6} M aminopterin, and 1.6×10^{-5} M thymidine. Transfections of HeLa and Hep-G2 cells were done with 10 μg of hMT-CAT plasmid DNA per dish (containing approximately 10^6 cells) for 4 h. The transfected cells were then shocked with 15% glycerol in 1× HEBS buffer (41) for 2 to 3 min. Cells were harvested 24 h after transfection. The CAT enzymatic activity was determined as described before (11).

RNA extraction and analysis. Total cytoplasmic RNA was extracted from several hMTK-I_B-transformed tk^+ colonies as described before (18). Samples (25 or 40 μg) of RNA were run on 1.5% agarose gels and blotted onto nitrocellulose membrane (38) or Gene Screen Plus hybridization transfer membranes (New England Nuclear Corp.). The blots were hybridized to nick-translated gene-specific probes. For S1 nuclease mapping of hMT-I_B mRNA, a probe was prepared by end labeling the unique *Bam*HI site at nucleotide +73 with [γ^{32}P]ATP, using T4 polynucleotide kinase (P-L Biochemicals, Inc.), and digesting with *Hin*fI (site at −87). The resulting 160-bp fragment was strand separated on an 8% strand-separating polyacrylamide gel. The specific activity of the probe was 5×10^6 cpm/pmol. For S1 analysis of hMT-II_A mRNA, a probe was made by end labeling a unique *Bam*HI site at nucleotide +76 as described above and then digesting with *Taq*I. The resulting 400-bp fragment was strand separated on a 5% strand-separating polyacrylamide gel. The specific activity was 6×10^5 cpm/pmol. A total of 30 μg of RNA (per sample) was hybridized overnight to 50,000 cpm of the corresponding probes in buffer containing 80% deionized formamide, 0.4 M NaCl, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.4, and 1 mM EDTA. S1 digestions were carried out with 300 U of enzyme (P-L Biochemicals, Inc.) per ml in a volume of 0.3 ml for 2 h at 37°C. The samples were resolved on a 6% polyacrylamide–7 M urea sequencing gel.

Isolation of DNA and Southern blotting. High-molecular-weight DNA was isolated from HeLa and Hep-G2 cells as described before (22). The restriction enzymes *Eco*RI, *Hpa*II, and *Msp*I were obtained from Boehringer Mannheim Biochemicals. DNA (10 μg) was digested with 3 U of enzyme per μg of DNA for 12 h, run on a 0.7% agarose gel, and transferred to nitrocellulose filters (Schleicher & Schuell, Inc.) as described before (37). To verify the completion of digestion, 500 ng of λ DNA was included in duplicate samples and visualized by staining with ethidium bromide. The filters were hybridized with a nick-translated probe derived from the 5′ leader and flanking region of the hMT-I_B gene (from *Hin*dIII to *Bam*HI).

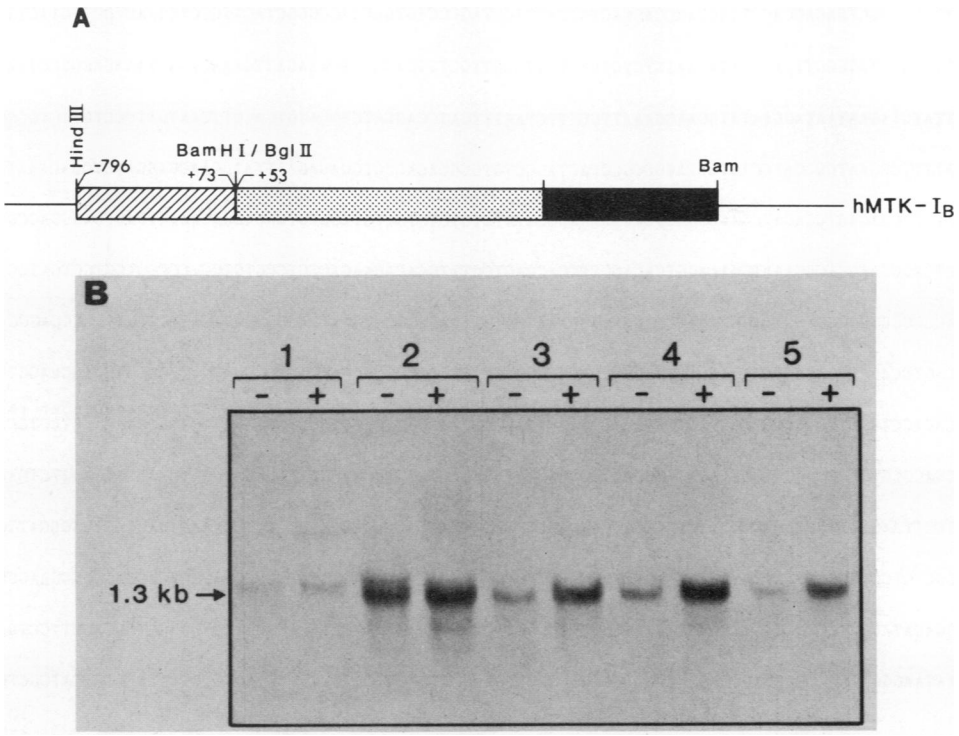


FIG. 2. Expression of the hMTK-I_B fusion gene. (A) Structure. Its construction is described in the text. Symbols: ▨, hMT-I_B DNA; ▩, herpes simplex virus coding sequences; ■, herpes simpex virus TK 3′ flanking sequences. Thin lines represent pUC8 sequences. The numbers indicate the nucleotide positions at the respective genes. (B) Expression in individual transfectants. A total of 12 tk^+ colonies were randomly picked and propagated into mass cultures. These cultures were incubated for 8 h in either normal growth medium (−) or in the presence of 5×10^{-6} M cadmium chloride (+). A total of 40 μg of total cytoplasmic RNA extracted from these cultures was analyzed by the Northern blot technique (38). The hybridization pattern of five representative clones, after probing the membrane with a TK-specific probe (29), is shown. The arrow indicates the size of TK mRNA. The filter was exposed for 16 h. As a control for the amount of RNA loaded on the gel, the same blot was hybridized to an actin probe, which did not reveal any significant differences (data not shown).

RESULTS

Structure of the hMT-I_B gene. The structure of the hMT-I gene cluster that contains the hMT-I_B gene is shown in Fig. 1A. The hMT-I_B gene is separated from the previously described hMT-I_A by two pseudogenes, ψhMT-I_C and ψhMT-I_D. The restriction map of hMT-I_B and the sequencing strategy are shown in Fig. 1B, and the complete nucleotide sequence of this gene is shown in Fig. 3. This gene contains two introns at exactly the same positions as the introns in other MT genes (10, 22, 32, 34). However, the introns in both hMT-I_A and hMT-I_B are larger (approximately 550 bp) than their counterparts in the mouse MT (mMT) genes or hMT-II_A. A TATAAA consensus sequence is present 29 bp upstream of the initiation site of transcription, as determined by the S1 nuclease protection technique (Fig. 4). Upstream from the TATA box, between nucleotides −54 and −41, there is the sequence TCTGCACCCCACCA, and further upstream, from nucleotide −114 to −127, the sequence ACTGCTCATGGCCC is found. Variants of these sequences are present in all MT genes described to date, located in at least two distinct regions, around nucleotides −150 to −130 and around the −50 region (4, 10, 18, 32, 34). These sequences correspond to the metal-responsive element (MRE) necessary for heavy metal inducibility (4, 22, 36).

The 61-amino-acid sequence derived from the hMT-I_B nucleotide sequence deviates from the published amino acid sequence of human MT-I (24) at four positions: Ala versus Thr at position 8, Cys versus Ser at position 32, Val versus Ile at position 49, and Ser versus Thr or Ala at position 53. The unique location of a cysteine residue at position 32 in hMT-I_B is of particular interest because of the involvement of cysteine residues in metal binding. While at this stage we do not know whether any of the amino acid differences have any significance regarding the function of the protein, the addition of a cysteine residue is a type of change expected to affect metal binding.

Cloned hMT-I_B gene has a functional metal-responsive promoter. To investigate whether the cloned hMT-I_B promoter was functional and to begin the characterization of its regulatory elements, we transfected an hMT-I_B-TK fusion gene (Fig. 2A) into *Rat2* tk^- fibroblasts. Expression of the fusion gene was induced by Cd^{2+} in most of the cell lines analyzed (Fig. 2B), indicating that the hMT-I_B gene has a functional metal-inducible promoter.

Expression of the endogenous hMT-I_B gene in human cells. We investigated the expression of the hMT-I_B gene in various cultured human cells to detect possible differences in basal expression and in cadmium responsiveness. For this purpose, total RNA was extracted from induced and uninduced HeLa and Hep-G2 (a human hepatoma cell line) (1) cells and from primary human foreskin fibroblasts. RNA transcripts were analyzed by the S1 nuclease protection technique (2) with a specific probe derived from the 5′ flanking and leader regions of the hMT-I_B gene. We also compared the expression of this gene with expression of the hMT-II_A gene, since the latter is known to be expressed in

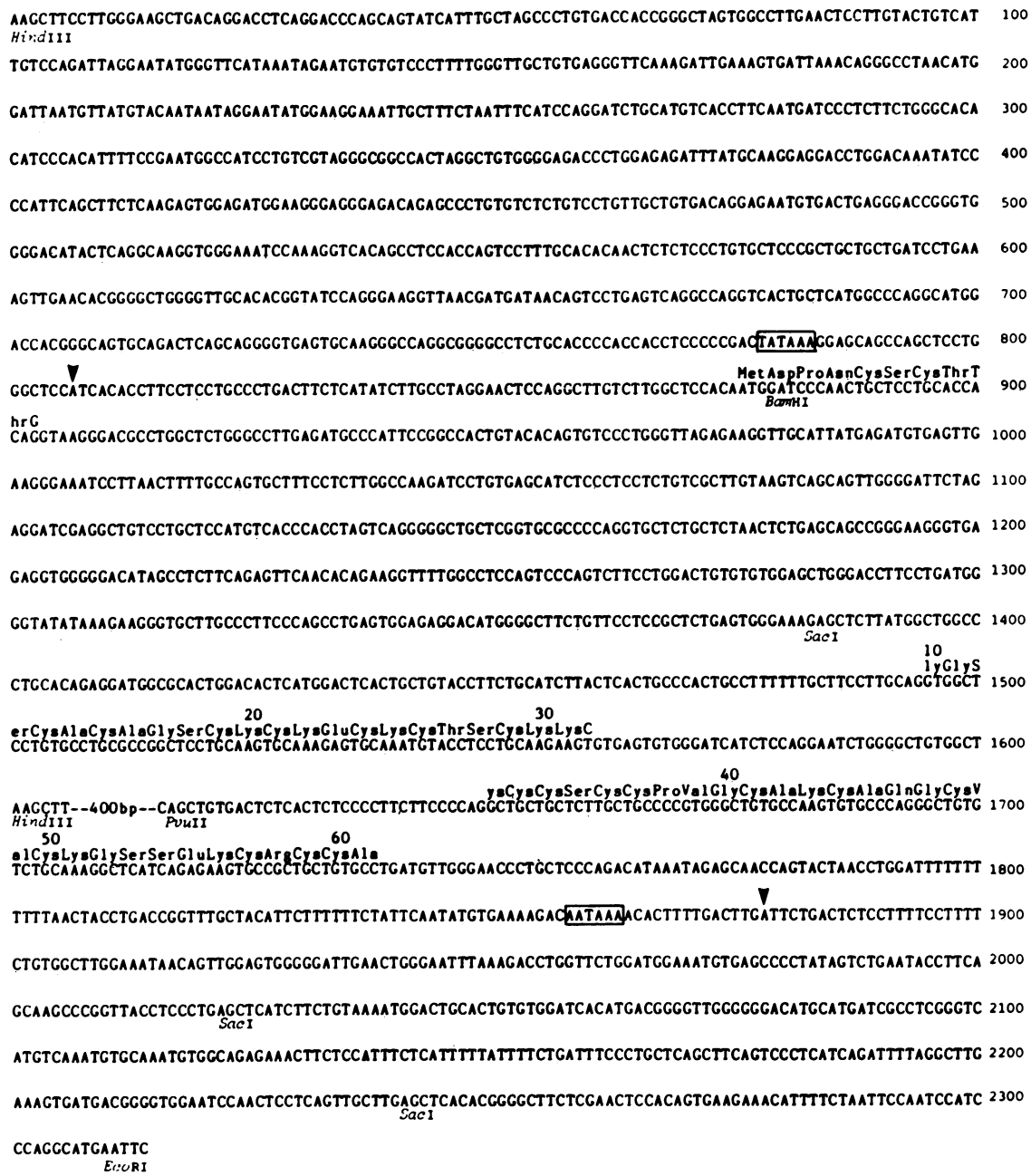


FIG. 3. Nucleotide sequence of the hMT-I_B gene region. The complete sequence of the 5′ flanking, coding, and 3′ flanking regions is shown. The gaps in the sequence correspond to short regions within the introns. The sequence was determined by the dideoxynucleotide method (33). TATAAA and AATAAA sequences are boxed. The initiation site of transcription is indicated by an arrow. Coding sequences are translated.

high levels, and its transcription is induced by Cd^{2+} in all cell lines we have tested (A. Heguy, G. Wong, and M. Karin, unpublished data). Figure 4 shows the results of such comparative S1 analysis. The hMT-II_A gene is expressed in high levels in all three cell types, and its mRNA levels are elevated by Cd^{2+} treatment. Interestingly, the basal level of hMT-II_A expression is considerably higher in the human fibroblasts than in the other cell types we have tested. In contrast, the hMT-I_B gene is expressed in high levels only in cadmium-induced Hep-G2 cells, although the absolute level of expression is at least fivefold lower than that of the hMT-II_A gene. The basal level is very low but detectable in longer exposures of the same gel (data not shown). No basal expression of hMT-I_B could be detected in either HeLa cells or primary cultures of human fibroblasts, even after very long exposures. However, in Cd^{2+} induced HeLa cells or primary fibroblasts, a low level of hMT-I_B-specific mRNA could be detected. This level is at least 10-fold lower than that detected in induced Hep-G2 cells. These results indicated that the hMT-I_B gene was preferentially transcribed in human hepatoma cells and suggested a cell type-specific expression.

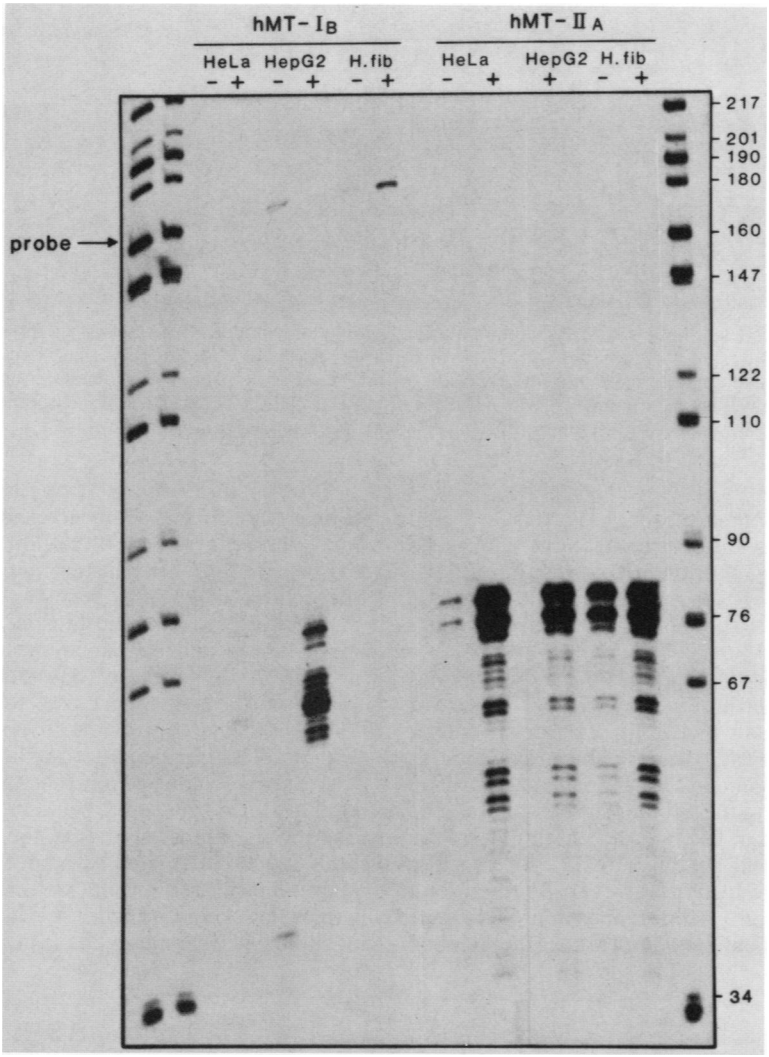


FIG. 4. Comparison of hMT-I_B and hMT-II_A gene expression by S1 nuclease analysis. Total cytoplasmic RNA from HeLa cells, Hep-G2 cells, and primary cultures of human fibroblasts was extracted after incubating the cells for 8 h in normal growth medium (−) or in the presence of 5×10^{-6} M $CdCl_2$ (+). RNA (40 μg) from each sample was analyzed for either hMT-I_B or hMT-II_A mRNA by the S1 nuclease protection technique. The length of the protected fragment is 73 bases for hMT-I_B and 76 bases for hMT-II_A. The *Hpa*II digestion products of pBR322 were used as size markers. The gel was exposed for 72 h.

To gain further knowledge on the cell type-specific expression of the hMT-I_B gene, we analyzed total cellular RNA from uninduced and induced human cell cultures by Northern hybridization. In addition to the cell lines examined above, we analyzed another hepatoma cell line (Hep-3B), two kidney carcinoma cell lines (A498 and A1212), and three B-cell lines (Loukes, Namalwa, and IB4). The Northern blots were hybridized with a probe specific for hMT-II_A, derived from the 3′ untranslated region of the phMT-II_3 cDNA clone (23) or with a hMT-I_B-specific probe derived from its 5′ untranslated region. The hMT-I_B-specific probe does not hybridize to the mRNAs of the hMT-II_A, hMT-I_A, or to other hMT-I genes expressed in HeLa cells. While the hMT-II_A gene was expressed in high levels in all of the cell lines (Fig. 5), expression of the hMT-I_B gene could be detected only in both liver cell lines (data for Hep-3B is not shown because it is identical to that obtained for Hep-G2) and in the two kidney-derived cell lines. Hybridization of the same blots with an hMT-I_A-specific probe revealed a pattern of expression similar to that of hMT-II_A, excluding the response to dexamethasone (data not shown). Like the hMT-I_A gene (32), the hMT-I_B 5′ flanking region does not contain a sequence resembling the glucocorticoid-responsive element present in the hMT-II_A regulatory region (20). In agreement with this observation, we could not detect induction of hMT-I_B mRNA in dexamethasone-treated renal carcinoma cells, while hMT-II_A mRNA was induced (Fig. 5). These results suggest that the hMT-I_B gene is preferentially transcribed in liver and kidney cells.

Tissue-specific expression of hMT-I_B is controlled in *cis*. The tissue-specific expression of the hMT-I_B gene could be due to the presence of tissue-specific promoter and regulatory elements recognized by liver- and kidney-specific *trans*-acting factors. Since in the past we found that the expression phenotypes of the hMT-II_A and hMT-I_A genes were controlled by their respective 5′ promoter/regulatory regions (32), we examined the 5′ flanking region of the hMT-I_B gene for the presence of tissue-specific regulatory and promoter elements by fusing it to a convenient reporter gene coding for CAT (11). The expression of the hMT-I_B-CAT chimeric

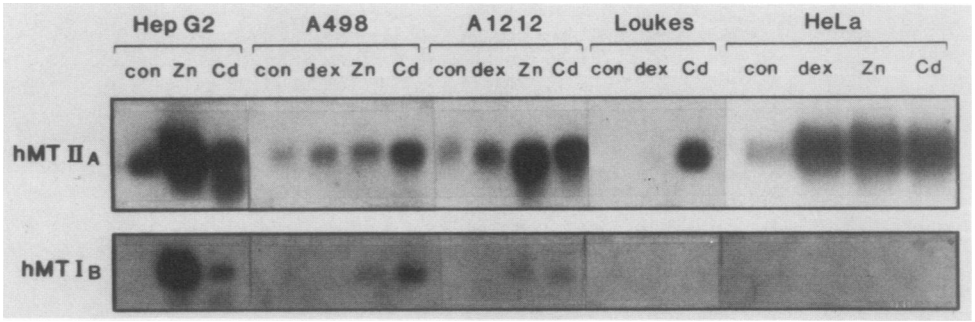
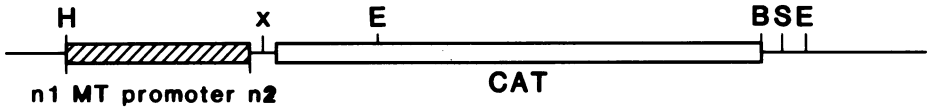


FIG. 5. Expression of hMT-I_B and hMT-II_A genes in human cells. Total cytoplasmic RNA from the cell lines indicated was extracted after a 12-h incubation with 10^{-6} M dexamethasone (dex)–2×10^{-4} M $ZnCl_2$ (Zn)–5×10^{-6} M $CdCl_2$ (Cd). RNA (25 μg) from each sample was analyzed by the Northern blot technique, using hMT-II_A-specific or hMT-I_B-specific probes as indicated. While hMT-II_A mRNA could be detected in all the cell lines, hMT-I_B-specific mRNA was present only in Hep-G2, A498, and A1212 cells. The autoradiogram shown in the upper panel is the result of a 12-h exposure; the one in the lower panel was exposed for 10 days.

gene was compared with that of the hMT-II_A-CAT and hMT-I_A-CAT fusion genes after transfections into cells which either express (Hep-G2) or do not express (HeLa) the endogenous hMT-I_B gene. To our surprise, the hMT-I_B-CAT fusion gene was expressed to the same relative extent in both cell lines (Fig. 6).

The lack of tissue specificity of expression of the hMT-I_B-CAT gene fusion could then be due to either of these two possibilities: (i) the elements which control tissue specificity of hMT-I_B gene are not present in its 5′ flanking region; or (ii) the expression of the endogenous hMT-I_B gene is controlled in *cis* by tissue-specific structural modifications of the gene itself. Since the expression of hMT-II_A and hMT-II_A is fully controlled by their 5′ flanking regions (20, 32), we consider the second alternative more likely. However, Southern blot analysis did not detect any gross differences in hMT-I_B gene copy number and arrangement between HeLa, Hep-G2, and primary fibroblasts (data not shown). We next considered methylation, a structural modification known to alter gene expression (14). To test the involvement of methylation in the control of hMT-I_B gene expression, we treated HeLa cells with 5′-azacytidine (5′azaC), an inhibitor of cytosine methylation, and examined the expression of the hMT-I_B and hMT-II_A genes by S1 nuclease protection analysis. Incubation with 10^{-6} M 5′azaC for 24 h led to a considerable increase in the expression of the Cd-induced hMT-I_B gene without affecting the pattern of hMT-II_A expression (Fig. 7).

A direct demonstration that the hMT-I_B gene is methylated in HeLa cells is provided in Fig. 8. *Msp*I versus *Hpa*II digestion demonstrated that the 5′ flanking region of the hMT-I_B gene, detected by a specific hybridization probe, is highly methylated in nonexpressing HeLa cells, but not in expressing Hep-G2 cells. We are currently mapping the positions of the methylated sites in the hMT-I_B gene by



MT·CAT

CATase ACTIVITY

Plasmid	Hela		Hep G2	
	-	+	-	+
phMT-I_B-CAT	50	311	49	236
phMT-I_A-CAT	176	835	80	402
phMT-II_A-CAT	135	3380	147	5040
pUCAT2	40	-	35	-

FIG. 6. Expression of transfected hMT-CAT genes in HeLa and Hep-G2 cells. The upper line represents the structure of the hMT-CAT constructions. Symbols: ▨, hMT promoter sequences; ▭, CAT transcription unit. Thin lines represent pUC13 sequences. Major restriction sites are indicated: H, *Hin*dIII; E, *Eco*RI; X, *Xba*I; B, *Bam*HI; S, *Sac*I. n_1 and n_2 the 5′ and 3′ borders, respectively, of the hMT 5′ flanking sequences present in the constructions: in phMT-I_B-CAT, n_1 is at −796 and n_2 is at +73; in phMT-I_A-CAT, n_1 is at −860 and n_2 is at +49; in phMT-II_A-CAT, n_1 is at −764 and n_2 is at +69. pUCAT2 was used as a negative control. CAT activities measured after transfection into either HeLa or Hep-G2 cells are expressed in picomoles of acetylchloramphenicol per milligram of protein per hour. Columns: (−), CAT activities in extracts from uninduced cells; (+), in cells induced with 5×10^{-6} $CdCl_2$ for 12 h. Since transfection efficiency varies with the cell type, only relative CAT activities should be compared.

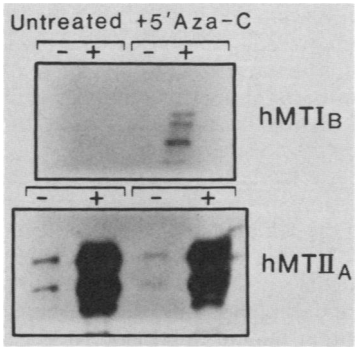


FIG. 7. Expression of hMT-I_B and hMT-II_A in 5′ azaC-treated HeLa cells. Exponentially growing HeLa cells (5×10^5 cells per dish) were treated with 10^{-6} M 5′azaC for 24 h, then incubated with fresh medium without (−) or with (+) 5×10^{-6} M $CdCl_2$ for 8 h, and harvested. Total cytoplasmic RNA (30 μg) was used in an S1 nuclease protection assay with either an hMT-I_B-specific probe or an hMT-II_A probe as indicated.

indirect end labeling, and we are investigating the methylation status of other neighboring hMT-I genes in different human cell lines and tissues.

DISCUSSION

Structure of the human MT-I_B gene. The substitution of a Gly in position 10 or 11 in the MT-I protein sequences for an Asp in MT-II is the only consistent difference between the two major forms of MT (15). Based on this criterion we placed the hMT-I_B gene in the MT-I subfamily because it has Gly codons at positions 10 and 11, as is the case for the hMT-I_A gene (32). The significance of these four differences between the deduced amino acid sequence of hMT-I_B and the published amino acid sequence of a mixture of at least three different MT-I proteins (24) is not known. However, the substitutions occur in positions which are probably not essential for heavy metal binding (3). On the other hand, the presence of an additional Cys residue at position 32 could have an effect on the metal-binding capacity of the protein. There are several forms of MT-I in human liver, and at least four of them are resolved by chromatographic procedures (16). Interestingly, although closely linked in the genome, the hMT-I_A and the hMT-I_B genes are quite divergent: the amino acid sequences predicted from their respective nucleotide sequences differ in 10 of 61 positions, suggesting that they encode functionally different MT-I isoforms.

The hMT-I_B gene has a functional promoter capable of rendering a heterologous gene responsive to cadmium induction, as we have demonstrated by gene fusion experiments. The 5′ flanking DNA contains at least two MREs, very similar to those identified for the mMT-I (4, 34, 36). mMT-II (34), hMT-II_A (20), and hMT-I_A genes (32). The MREs are highly conserved in all MT genes so far described, and mutagenesis experiments (4, 20, 36) have demonstrated that the presence of this conserved sequence is necessary for metal induction. However, a formal proof of the role of the MREs in mediating hMT-I_B Cd^{2+} responsiveness would require mutational analysis as described above.

Cell type-specific expression of hMT-I_B. Since there are several isoforms of hMT-I protein and several genes encoding these variants, it was interesting to investigate whether these genes were expressed preferentially in different cell types. We favored the hypothesis that different MT-I genes are expressed in different tissues, because this could explain

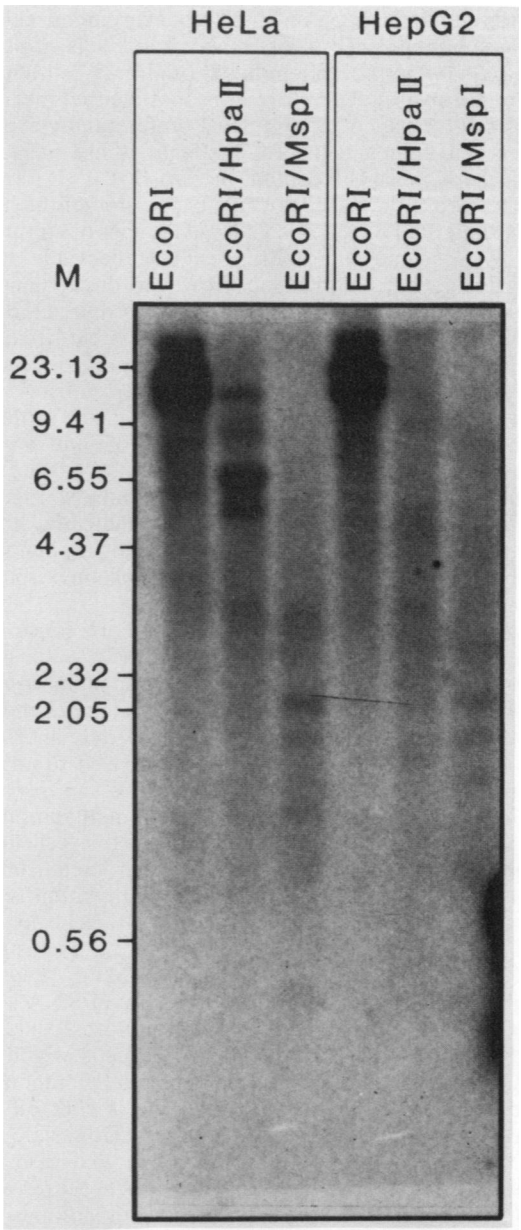


FIG. 8. Tissue-specific differences in methylation of the 5′ flanking region of the hMT-I_B gene. DNA was extracted from HeLa and Hep-G2 cells, and 10-μg samples were digested with either *Eco*RI alone, *Eco*RI plus *Msp*I, or *Eco*RI plus *Hpa*II. The digested DNA was separated electrophoretically and transferred onto nitrocellulose filters which were probed with the 5′ flanking region (*Hin*dIII-*Bam*HI) of the hMT-I_B gene. M, Position and sites of *Hin*dIII cleavage products of λ DNA used as a marker. *Msp*I and *Hpa*II share the same recognition site, 5′CCGG, but *Msp*I is not sensitive to CpG methylation, and *Hpa*II is sensitive.

the multiplicity of MT-I genes and would be a way to increase the potential for differential and cell type-specific regulation of Zn and Cu metabolism (17). We have previously shown that both hMT-II_A and hMT-I_A genes are expressed in HeLa cells and human fibroblasts, although their regulation is different, and the level of expression is at least five times lower for the hMT-I_A gene (32). The hMT-I_B gene also is expressed in approximately a fivefold-lower level than hMT-II_A, but unlike hMT-I_A, hMT-I_B is expressed

primarily in human hepatoma and renal carcinoma cell lines. This gene is expressed at extremely low levels, if at all, in HeLa cells (which are of epithelial origin), B-cell lines, and primary fibroblasts. Therefore, it is possible that the hMT-I_B gene is cell type specific, or at least preferentially expressed in liver and kidney cells. Interestingly, these tissues also exhibit the highest MT content in vivo (15).

At this point, the tissue-specific expression of the hMT-I_B gene is suggested by studying a large number of cultured cell lines. We assume that these results reflect the in vivo expression patterns of the gene. However, direct demonstration of tissue-specific expression in vivo requires the analysis of human tissues. This is nearly impossible because the basal level of expression of hMT-I_B, even in Hep-G2 cells, is very low, and hMT-I_B mRNA can be clearly observed only after induction by Cd^{2+}. We hope to solve this problem by performing studies on primary cultures of human organs. However, we have shown that hMT-I_B transcripts are absent not only in certain transformed cell lines but also in primary cultures of human foreskin fibroblasts, indicating that the tissue-specific expression of hMT-I_B is not a phenomenon arising from the long-term passage of cell lines, but is of physiological relevance.

Unlike other liver-specific genes which are controlled by *trans*-acting factors specific to liver cells (5, 7, 30), hMT-I_B tissue-specific expression is not determined in *trans*. An hMT-I_B-CAT fusion is expressed both in Hep-G2 and HeLa cells; the endogenous gene is expressed only in Hep-G2. Since the expression of hMT-I_B is increased dramatically after incubation of HeLa cells with 5'azaC, an inhibitor of cytosine methylation (14), it appears that methylation keeps the hMT-I_B gene in a nonactive state in this cell line and probably in others. In fact, when DNA extracted from HeLa cells is analyzed by digestion with a methylation-sensitive enzyme, the 5' flanking region of the hMT-I_B gene appears to be much more extensively methylated in these cells than in Hep-G2 cells. The very low level of hMT-I_B expression which can be detected after long exposure of RNA samples from Cd-induced HeLa and fibroblast cultures could therefore be due to a small subpopulation of cells which fail to methylate their hMT-I_B gene. Compere and Palmiter (6) have previously shown that the mMT-I gene is shut off in W7 thymoma cells by cytosine methylation. However, in that case the methylation was not selective and also led to inhibition of mMT-II gene expression (28). Moreover, the inhibition of mMT gene expression by methylation is unique to the W7 cell line (R. Palmiter, personal communication). On the other hand, the hMT-I_B gene is not active in the majority of the cell lines and primary cultures that we have examined, and the closely linked hMT-I_A and hMT-II_A genes are not affected. We are currently investigating which regulatory sites on the hMT-I_B gene are specifically affected by methylation and whether the same elements are present in the hMT-I_A and hMT-II_A genes. In any case the current results suggest the existence of a novel mechanism for control of gene activity within a multigene family by gene-specific and tissue-specific methylation events.

Although the mechanisms involved in control of tissue-specific gene expression by methylation are not yet clear, the hMT-I_B gene represents the first example of a tissue-specific MT gene. It is possible that in multigene families which arose by recent duplication events, like the hMT family, some of the members have not yet sufficiently diverged to allow recognition of their promoter/regulatory elements by different factors. In such a case, controlled methylation and demethylation events may constitute a mechanism for tissue-specific expression which does not require new specificities of promoter recognition by tissue-limited regulatory factors. It would be interesting to investigate the possibility of a correlation between methylation of specific sites in the control region of hMT-I_B and the binding of specific transcription factors. As discussed earlier (17), different tissues and cell types are likely to have different requirements for zinc and copper ions, whose availability is controlled by MT. Understanding the full physiological significance of selective expression of the hMT-I_B gene and the amino acid differences of its cognate protein will require purification of the latter protein and comparison of its biochemical properties with those of other human MT-I isoforms.

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LITERATURE CITED

1. **Aden, D. R., A. Fogel, S. Plotkin, I. Damjanov, and B. B. Knowles.** 1979. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature (London) **282**:615–616.
2. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
3. **Boulanger, Y., C. M. Goodmann, C. P. Forte, S. W. Fesik, and I. M. Armitage.** 1983. Model for mammalian metallothionein structure. Proc. Natl. Acad. Sci. USA **80**:1501–1505.
4. **Carter, A. D., B. K. Felber, M. J. Walling, M. F. Jubier, C. J. Schmidt, and D. H. Hamer.** 1984. Duplicated heavy metal control sequences of the mouse metallothionein-I gene. Proc. Natl. Acad. Sci. USA **81**:7392–7396.
5. **Ciliberto, G., L. Dente, and R. Cortese.** 1985. Cell-specific expression of a transfected human α_1-antitrypsin gene. Cell **41**:531–540.
6. **Compere, S. J., and R. D. Palmiter.** 1981. DNA methylation controls the inducibility of the mouse metallothionein I gene in lymphoid cell. Cell **25**:233–240.
7. **D'Onofrio, C., V. Colantuoni, and R. Cortese.** 1985. Structure and cell-specific expression of a cloned human retinol binding protein gene: the 5' flanking region contains hepatoma specific transcriptional signals. EMBO J. **4**:1981–1989.
8. **Friedman, R. L., S. P. Manly, M. McMahon, I. M. Kerr, and G. R. Stark.** 1984. Transcription and posttranscriptional regulation of interferon induced gene expression in human cells. Cell **38**:745–755.
9. **Friedman, R. L., and G. R. Stark.** 1985. α-Interferon-induced transcription of HLA and metallothionein genes containing homologous upstream sequences. Nature (London) **314**:637–639.
10. **Glanville, N., D. M. Durnam, and R. D. Palmiter.** 1981. Structure of mouse metallothionein-I gene and its mRNA. Nature (London) **292**:267–269.
11. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
12. **Griffith, B. B., R. A. Walters, M. D. Enger, C. E. Hildebrand,**

and J. K. Griffith. 1983. cDNA cloning and nucleotide sequence comparison of Chinese hamster metallothionein I and II mRNA's. Nucleic Acids Res. **11**:901–910.
13. **Gronenborn, B., and J. Messing.** 1978. Methylation of single-stranded DNA *in vitro* introduces new restriction endonuclease cleavage sites. Nature (London) **272**:315–317.
14. **Jones, P.** 1985. Altering gene expression with 5′ azacytidine. Cell **40**:485–486.
15. **Kagi, J. H. R., and M. Nordberg, ed.** 1979. Metallothionein. Birkhauser Verlag, Basel.
16. **Kagi, J. H. R., M. Vasak, K. Lerch, D. E. O. Gilg, P. Hunziker, W. R. Bernhard, and M. Good.** 1984. Structure of mammalian metallothionein. Environ. Health Perspect. **54**:93–103.
17. **Karin, M.** 1985. Metallothioneins: proteins in search of a function. Cell **41**:9–10.
18. **Karin, M., R. D. Anderson, E. Slater, K. Smith, and H. R. Herschman.** 1980. Metallothionein mRNA induction in HeLa cells in response to zinc or dexamethasone is a primary induction response. Nature (London) **286**:295–297.
19. **Karin, M., R. L. Eddy, W. M. Henry, L. L. Haley, M. G. Byers, and T. B. Shows.** 1984. Human metallothionein genes are clustered on chromosome 16. Proc. Natl. Acad. Sci. USA **81**: 5494–5498.
20. **Karin, M., A. Haslinger, H. Holtgreve, R. I. Richards, P. Krauter, H. M. Westphal, and M. Beato.** 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-II_A gene. Nature (London) **308**:513–519.
21. **Karin, M., R. Imbra, A. Heguy, and G. Wong.** 1985. Interleukin I regulates human metallothionein gene expression. Mol. Cell. Biol. **5**:2866–2869.
22. **Karin, M., and R. I. Richards.** 1982. Human metallothionein genes: primary structure of the metallothionein-II gene and a related processed pseudogene. Nature (London) **299**:797–802.
23. **Karin, M., and R. I. Richards.** 1982. Human metallothionein genes: molecular cloning and sequence analysis of the mRNA. Nucleic Acids. Res. **10**:3165–3173.
24. **Kissling, M. M., and J. H. R. Kagi.** 1979. Amino acid sequence of human hepatic metallothionein, p. 145–151. *In* J. H. R. Kagi and M. Nordberg (ed.), Metallothionein. Birkhauser Verlag, Basel.
25. **Kushner, I.** 1982. The phenomenon of the acute phase response. Ann. N.Y. Acad. Sci. **389**:39–45.
26. **Laimins, L., G. Khoury, C. M. Gorman, B. H. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA **79**:6453–6457.
27. **Le Beau, M. M., M. O. Diaz, M. Karin, and J. D. Rowley.** 1985. Metallothionein gene cluster is split by chromosome 16 rearrangements in myelomonocytic leukemia. Nature (London) **313**:709–711.
28. **Lieberman, M. W., L. R. Beach, and R. D. Palmiter.** 1983. Ultraviolet radiation-induced metallothionein-I gene is associated with extensive DNA demethylation. Cell **35**:207–214.
29. **McKnight, S. I.** 1980. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. Nucleic Acids Res. **8**:5949–5964.
30. **Ott, M. O., L. Sperling, P. Herbomel, M. Yaniv, and M. C. Weiss.** 1984. Tissue specific expression is conferred by a sequence from the 5′ end of the rat albumin gene. EMBO J. **3**:2505–2510.
31. **Ricca, G. A., J. W. McLean, and J. M. Taylor.** 1982. Kinetics of induction of α_1-acid glycoprotein. Ann. N.Y. Acad. Sci. **389**:88–103.
32. **Richards, R. I., A. Heguy, and M. Karin.** 1984. Structural and functional analysis of the human metallothionein I_A gene: differential induction by metal ions and glucocorticoids. Cell **37**:263–272.
33. **Sanger, F., F. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
34. **Searle, P. F., B. L. Davison, G. W. Stuart, T. M. Wilkie, G. Nordsted, and R. D. Palmiter.** 1984. Regulation, linkage, and sequence of mouse metallothionein I and II genes. Mol. Cell. Biol. **4**:1221–1230.
35. **Stearman, R. S., C. A. Lowell, W. R. Pearson, and J. F. Morrow.** 1982. Regulation of synthesis of amyloid a-related protein. Ann. N.Y. Acad. Sci. **389**:106–115.
36. **Stuart, G. W., P. F. Searle, H. Y. Chen, R. I. Brinster, and R. D. Palmiter.** 1984. A 12-base-pair motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. Proc. Natl. Acad. Sci. USA **81**:7318–7322.
37. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
38. **Thomas, P.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
39. **Thornalley, P. J., and M. Vasak.** 1985. Possible role for metallothionein in protection against radiation induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim. Biophys. Acta **827**:36–44.
40. **Vieira, J., and J. Messing.** 1983. The pUC plasmids, an M13mp-7-derived system for insertion, mutagenesis and sequencing with universal primers. Gene **19**:259–268.
41. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell **16**:777–785.